

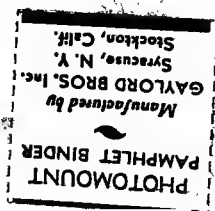
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January 30, 1913

A STUDY OF EPITHELIOMA CONTAGIOSUM OF THE COMMON FOWL

BY

CLIFFORD D. SWEET

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OF THE COMMON FOWL*

BY

CLIFFORD D. SWEET

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INTRODUCTION

Economic Importance.—From an economic standpoint epithelioma contagiosum is a disease of considerable interest. In certain localities, notably in the Hawaiian Islands, and South Africa, it is one of the chief obstacles to the profitable raising of poultry. It attacks young fowls, is very contagious and proves fatal in a large percentage of the fowls attacked. In California it is apparently less widespread and seemingly has not so great a mortality percentage as in the above-mentioned countries. However, even here, a clear understanding of the epidemiology of the disease, with the control such a knowledge would give of its incidence and spread, would be of very considerable economic value.

* Submitted to the Department of Zoology, in partial fulfillment of the requirements for the degree of Master of Science, in the University of California, April 22, 1912.

Scientific Importance.—In addition to its economic significance, epithelioma contagiosum is a disease of unusual scientific interest, not only because of the obscure nature of its etiology, but because of its apparent relationship or resemblance to other diseases. Because of the epithelial hyperplasia, which is one of the most striking features of the disease, there has been a tendency to classify the lesions produced as neoplasms. Because of the presence of a filterable virus and cell inclusions resembling those of human molluscum contagiosum, variola and vaccinia, it has been classified as one of the exanthemata, and its exciting agent reputed to be a protozoan, in common with many other diseases of this class. Again many others as Friedberger and Fröhner (1908), Schmid (1909), Sigwart (1910), and Kinsley (1907) assert that epithelioma contagiosum and avian diphtheria are different manifestations of the same disease, varying only in clinical features and in nature of, or severity of infection with the same causative agent. This latter contention is based on the fact that in many cases fowls present the clinical features of both diseases simultaneously, and that by inoculation with material taken from chickens having apparently one disease, both diseases may be produced.

With the hope of throwing some further light on the cause and nature of this most interesting disease, the following study was attempted. It is necessarily incomplete and leaves many points requiring further investigation. However, perhaps others may be led to give their attention to further studies of this disease, because of some of the data here presented. The investigations here reported were carried on under a grant from the Federal Adams fund, of the Agricultural Experiment Station of the University of California, for which grateful acknowledgments are here made. The work has been under the direction of Professor Charles A. Kofoid, of the Department of Zoology, and Assistant Professor Clarence M. Haring, of the Veterinary Division of the Agricultural Department, for whose many suggestions and criticisms I am deeply indebted. I should also like to acknowledge the kindly interest shown by Assistant Professor G. Y. Rusk, of the Department of Pathology, and the continual

help I received from my friend, Mr. O. H. Robertson, who was working in the same laboratory on a somewhat similar problem.

History of Disease.—An historical review of the disease hardly seems necessary since it has already been so well done by Reischauer (1906), Burnet (1906), and others. Only a few of the main points will be mentioned in passing. Rivolta (1865) described cellular inclusions, similar to those already described by Virchow in *molluscum contagiosum* of man. These inclusions he interpreted as gregarines. This was also the classification of Friedberger and Fröhner (1908), who described avian diphtheria as (1) croupous diphtheritic mucous membrane inflammation, the familiar diphtheria of fowls, bacterial in origin, and (2) croupous diphtheritic mucous membrane inflammation in which the mucous membranes of the head give the same clinical picture as in the diphtheria of bacterial origin, but in addition *epithelioma contagiosum* may appear on the skin of the head, a condition ascribed to the presence of gregarines. Bollinger (1873) characterized the disease as an epithelioma. Marx and Sticker (1902) proved the causative agent of the disease to be filterable through a Berkfeld filter (but not through a Chamberland F filter) and thereby placed it in the great class of diseases which are supposed to be due to ultra-microscopic organisms. They also demonstrated the very great resistance of the virus to heat and chemical agents, finding the dried virus still virulent after being placed in sealed tubes, within a steam sterilizer at 100° C, for an hour. After thirty minutes' exposure to 2% carbolic acid the virus was no longer active, but was active after very long exposure to the action of 1% carbolic acid. They conclude that the cell inclusions are the products of cell degeneration produced by an ultra-microscopic, very resistant virus. Juliusberg (1904) confirmed the filtration results of Marx and Sticker (1902). Borrel (1904) observed granules within the cell inclusions and thought the inclusions were the organic causative agent of the disease. Reischauer (1906) made extensive microscopic studies of the diseased tissue and was of the opinion that the cell inclusions contained the causative agent of the disease and represented development stages of a protozoan. However, it does not seem that he was able to demonstrate a complete life cycle, although

certain of his figures (for example fig. 25) are very suggestive of stages in the development of an organism. He regarded epithelioma contagiosum and avian diphtheria as separate diseases and held that the lesions found on the mucous membranes of the mouth, nasopharynx, and throat in epithelioma contagiosum were extensions to these surfaces of the lesions found on the skin. Bur-net (1906) made a study of the morphology of the disease, but came to no definite conclusion from it. In common with earlier writers he placed the period of incubation at about five or six days. He found the blood and internal organs of infected fowls contained the virus, as shown by the fact that he was able to reproduce the disease by inoculation of healthy fowls with the blood and liver of diseased ones. He also produced the disease by allowing fowls to ingest the virus, and by intravenous injection, in each of which methods the lesions appeared at the usual points of election and not elsewhere, except in one case where a typical lesion was found at post mortem in the oesophagus of a fowl which had eaten the virus.

CLINICAL FEATURES

The clinical features of the disease have also been described at length by many writers and we will but sketch them here, making reference chiefly to a few facts in our observations which supplement those recorded by others.

Period of Incubation.—As has been generally noted, the period of incubation is five or six days. However, this varies with the mode of infection and the virulence of the infective material. With virus which had not been attenuated in any way lesions often appeared on the comb and wattles in three days or even sooner after scarification. The same virus when injected intravenously required a longer period of incubation. On the other hand, virus which had been attenuated by age, heat, or chemicals required a greatly lengthened period in which to manifest itself in a macroscopic lesion. These variations in the length of the incubation period are shown in Table I, with the history of the virus used in each case.

TABLE I

History of Virus	Incubation period in days	Growth	Number of fowls inoculated
1. Kept in test tube closed with cotton plug from May, 1909, to July, 1910	7	Small	5
2. Virus used in above after one passage through fowl	(a) 3 (b) 4	Good	(a) 8 (b) 3
3. Kept in test tube closed with cotton plug from May, 1906, to August, 1910	10	Slight	3
4. Mixture of viruses. (a) Kept in test tube closed with cotton plug from May, 1906, to July, 1911, (b) Kept in same manner from August, 1910, to July, 1911	5 & 6	Small and increased in size slowly	4
5. Virus used in No. 4 after one passage through fowl	(a) 4 (b) 6	Fair	(a) 3 (b) 3
6. Virus used in No. 5 after an additional passage through a fowl	(a) 4 (b) 3	Abundant	(a) 6 (b) 1
7. Virus used in No. 6 after an additional passage through a fowl	3	Abundant	6
8. Powdered virus kept in open mortar from August, 1910, to July, 1911	Result, negative		3
9. Virus received from Honolulu—freshly removed from infected fowls	3		6
10. Some virus in No. 9, given intravenously	8 to 12	Lesion appearing first on mucous membranes	3
11. Virus used in No. 7, given intravenously	10 to 12		3
12. Blood from infected fowl, injected intravenously	10	Growth in throat and on comb	1

In those cases in which the incubation period was greatly lengthened by an attenuation of the virulence of the virus, the rate of growth was much slower, although the ultimate course of the disease seemed to be but little changed, this being in accord with the observations of Burnet (1906).

Clinical Course.—When the disease is inoculated by scarifying the comb and wattles there appear after varying periods of inoculation, small, raised, whitish granulations at the points of inoculation. In the early stages these granulations bear a considerable resemblance to tubercles, but later become larger, firmer, more circumscribed, and form a cauliflower-like tumor increasing in size up to about the tenth day. On the tenth to the twelfth day dry scabs form on the surface of the growth, and in the following days the whole growth gradually hardens into a crust, becomes smaller, and at the end of two or three weeks is readily

removed or falls off leaving a core of granulation tissue. This core soon shrivels and disappears, leaving little or no scar.

Intravenous Inoculation.—When inoculation is by intravenous injection, the period of incubation is greatly lengthened, but otherwise the clinical course is unchanged.

Mucous Membrane Inoculation.—If the mucous membranes of the mouth be inoculated small spongy masses form at the point of inoculation. These break down to form cheesy masses (probably from secondary infection) and in some measure resemble the membrane of diphtheria. In about one half our cases these patches appeared on the mucous membranes of the mouth and throat following an inoculation by scarification of the comb, although the mucous membranes were left entirely intact in the operation. In about an equal number of cases the disease ran its entire course without the mucous membranes showing any change whatever (inoculation being by scarification of the comb and wattles).

Relationship of Virulence of Virus to Resistance of Host.—It would seem that the occurrence of lesions is, or is not limited to the point of inoculation by the relation which exists between the resistance of the host and the virulence of the virus used. Naturally such a relationship is difficult to determine, but our results are at least strongly suggestive of its existence. If a small amount of a weakened virus was used and the area of sacrifice was small, the lesion produced did not spread beyond the original area of scarification, and remained small throughout the course of the disease which was somewhat shortened. If a large amount of the same weakened virus was used, but the area scarified was large, a large growth was produced, although in no case was a growth noted except at the seat of the original inoculation. When the infective agent has a lowered virulence the period of incubation is prolonged, the manifestation of the disease is limited entirely to the site of ingress, the course of the disease may be shortened, and there is no clinical evidence of any general constitutional effect except an immunity to further infection with the same agent. It seems that this observation is of considerable importance in determining the nature of the disease and must be taken as a strong reason for believing epithelioma contagiosum to be an infectious disease.

Mildness of Inoculated Disease.—In a series of over fifty inoculations, where the virus was unmixed with roup, no fatal terminations were seen except in two cases, in which the vitality was greatly lowered by excessive bleeding. These two fowls became moribund and were killed. At autopsy the internal organs were normal as were the mucous membranes of the head and throat. Cultures taken from the depths of the growths on the comb and from the ventricles of the heart, gave a growth of an organism that was probably *Staphylococcus albus*. This would suggest that death was due to a terminal infection, the portal of entry being the lesions on the head. These findings conflict with those of Reischauer (1906), who found the heart blood sterile in a considerable number of cases, and are too few in number to be more than suggestive. With the exception of these two cases, at no time during the course of the disease were the fowls very much disturbed, as they continued to take food and in every other way to act normally. As Cary (1906) and others have pointed out, the disease is not often fatal except in young fowls. The fowls we used were for the most part nearly grown. However, our records lead us to believe that the disease is a much milder one than roup and that much of the mortality which has been attributed to it may be due to the simultaneous occurrence of the two diseases. Of course, it is also possible that our percentage of mortality was lowered by the virus used (although several were used) being less active than that used by others, or it may be that the climate at Berkeley is unfavorable to the development of the disease. The fact that a number of strains of virus were used would render the first supposition improbable and we can hardly believe too great weight should be given the latter factor.

Epidemiology.—*Epithelioma contagiosum* is evidently contracted directly. Burnet (1906) has shown that it can be contracted by ingestion of the virus, that the virus is present in the blood stream, and that the typical disease can be produced by intravenous injection. We have confirmed his experiments showing the virus to be present in the blood stream, by injecting blood from a diseased fowl into a normal fowl, and thereby producing specific lesions, not only on the mucous membranes, but also at the usual sites on the comb and wattles. We are

inclined to believe the infection spreads largely by the ingestion of virulent material, as we have seen fowls eat the scabs which fall to the ground and also pick them from the heads of their infected neighbors.

PATHOLOGY

Introduction.—The etiological agent of epithelioma contagiosum has long been thought to be contained in the cell inclusions mentioned by every one who has studied the disease. There is no question but that the causative agent is contained in the epithelial growths since the disease is so readily transmitted by the introduction of even a very minute quantity of the growth into a healthy fowl. Since, from the standpoint of its transmissibility, the disease is so evidently infectious, one would naturally expect to find a specific organism in the lesions produced.

With the above idea in mind, one is naturally struck by the constantly appearing cell inclusions in the epithelial overgrowths. Very similar cell inclusions appear in the cutaneous lesions of variola, vaccine, and human molluscum contagiosum, and are regarded by some as the causative agents of the disease, it being held that they are protozoan in character. Councilman (1904) and his associates have especially emphasized these points, a life cycle having been worked out for *Cytorcytes variolae* by Calkins (1904). In the hope of being able to discover the nature of the cell inclusions in epithelioma contagiosum we examined about four hundred sections from the lesions appearing on the head and on the mucous membranes of the mouth and throat. These sections were taken at varying intervals, mostly from the third to the twelfth day. They were fixed in Zenker's fluid and hardened by the usual methods and stained by the following methods: (1) hematoxylin and eosin; (2) Giemsa's blood stain; (3) Heidenhain's iron haematoxylin and Bordeaux red; (4) Mühlens-Hartmann's vaccine stain; (5) von Wasielewski's stain; (6) Mann's eosin-methylene blue; (7) Osmic acid; (8) Sharlach R; and (9) Levaditi's silver impregnation method. Also a large number of smears were made directly from the tumor growths, fixed with methyl alcohol and stained by the Giemsa method.

Tissue Reaction.—The tissue changes described by Reischauer (1906), Burnet (1906), Apolant (1902), and many others, were noted. These are mainly as follows: There is a marked hyperplasia of the epithelium, the number of cells being greatly increased, and the individual cells being greatly enlarged. The cells apparently increase in number by the usual method, that is, the cells multiply in the stratum germinativum by mitosis and in the portions of the growth removed from the growing layer undergo only secondary changes. It also appears that the growing layer is unequally stimulated, since the proliferating epithelium takes the form of cell nests or buds projecting downward into the underlying connective tissue and upward to form the cauliflower-like growths which appear on the surface of the epithelium. These buds or fingers of epithelial cells, on section, very much resemble in appearance a true epithelioma. They are supported by thin strands of connective tissue, there being considerable hyperplasia of the underlying connective tissue. In the advanced stages of the disease this hyperplasia of connective tissue elements is much more marked. However, the excess connective tissue may in large part be due to the secondary infection which is always present. In addition to the above noted changes there is a marked engorgement of the underlying blood vessels and a considerable wandering of leucocytes into the tissue (including the epithelium) lying about the vessels. Even, in the early stages, before secondary infection has apparently taken place to any marked degree there is the tissue reaction that is present about a focus of infection. In other words, the nature of the tissue reaction speaks strongly for the infectious nature of the disease, varying from the picture presented by the ordinary pyogenic infection only in the unusual reaction of the epithelium. As has been noted, there is an enormous increase in the number of epithelial cells. Besides this, the individual cells become as large as $40\ \mu$ in diameter. The epithelial cells lying next the connective tissue are about normal in size, stain deeply and are undergoing rapid proliferation. Their walls are well defined, the cytoplasm dense and finely granular, and the nuclei are normal in appearance. The cells outside the deepest layers in the epithelial bud become progressively larger, stain less

deeply, the cytoplasm becomes pale and less granular; being vacuolated in many cells, the cell walls become less well marked and finally disappear so that the central portion of the nest is a necrotic mass of indistinct cell elements. In the same order, the nuclei are also enlarged, become increasingly vacuolated, contain bizarre clumps of chromatin, stain faintly, the limiting membrane becomes less well defined and finally disappears entirely so that they (the nuclei) are no longer visible.

Cell Inclusion.—Beside the normal cell contents nearly all of the epithelial cells contain the cell inclusion mentioned above. This cell inclusion which occurs so constantly in the epithelial cells, has been thought by many to be analogous to the cell inclusion present in carcinomata and by others to the inclusion seen in the lesions of variola, vaccina, etc. Reischauer (1906) and others have taken it to be an animal parasite, while Burnet (1906), Apolant (1902), and many others thought it to be merely a product of cell degeneration.

Form and Occurrence in Cells.—The cell inclusion is spheroidal or ellipsoidal, usually larger than the nucleus, has fairly definite boundaries, although we were not able to see a definite limiting membrane. It lies in the neighborhood of the nucleus. Usually a single one is found in each cell, the exceptions to this being, first, at times two of these bodies are seen in a single cell; and second, some enlarged cells do not appear to contain any. However, this apparent absence of a cell inclusion is probably due to the inclusion lying outside the plane of the section in the enlarged epithelial cell. Rarely these bodies are seen in intercellular spaces. Apparently these cell inclusions are not present in the epithelial cells which are in process of active proliferation. However, this is a difficult point to determine accurately, because there are cells containing the cell inclusion which lie within the proliferating zone and which do not show any signs of degeneration. Although these cells do not show any signs of degeneration, only those which do not contain the cell inclusion show evidence of normal active proliferation. At times cells containing the cell inclusion also contain atypical mitotic figures, but on the whole, it seems fairly certain that the appearance of the inclusion in a cell accompanies a cessation of proliferation.

Nature of Inclusion.—It seems that epithelioma contagiosum produces a very rapid proliferation of epithelial cells, those cells which are in a state of proliferation remaining free from visible cell inclusions. This would suggest that this cell inclusion, in its enlarged state at least, appears in a cell only after its birth as an independent cell, and that it is only after the cell is no longer proliferating that the cell inclusion appears. This would at once lead one to label the inclusion as a product of cell degeneration, as Burnet (1906), Apolant (1902), Marx and Sticker (1902) have done for this disease, and as Ewing (1904) has done for the inclusions seen in vaccina. However, the fact that in the neighborhood of the proliferating zone many cells appear which contain well marked cell inclusions, but which show little or no evidence of degeneration, makes one hesitate to make so absolute a conclusion. So if one assumes that the cell inclusion is merely a product of cell degeneration he must assume that it is due to some chemical change within the cell which does not manifest itself as a usual type of degeneration until late in the life of the cell. This does not seem a very difficult assumption to make in view of the very extensive changes produced in the epithelial elements.

Changes in Inclusions.—As mentioned above, in passing from the proliferating zone inward to the center of the epithelial cell nests, progressive degeneration of the cytoplasm and nucleus are seen. Apparently, the same progressive degeneration is seen in the cell inclusions. In the region of the proliferating zone the cell inclusions stain deeply, and are clear cut in outline, but as one goes away from this zone toward the center of the epithelial bud they stain less deeply, have less clearly defined margins, become increasingly vacuolated and finally are completely disintegrated. Here again one is tempted to ascribe all this change to progressive degeneration, but is deterred by remembering that it so very closely follows the normal sequence in the life cycle of protozoan cell parasites. Very often there appear smaller bodies within the cell inclusions, giving them something of a mulberry appearance. This occurs often enough and is so well marked that it very strongly suggests the presence of a stage of sporulation. Also in fixed preparations stained with Giemsa

stain and with hematoxylin and eosin, as well as in fresh smears stained in the same manner, there are small acid staining granules within the epithelial cells which suggest a stage of fragmentation. However, we are not able, at this time, to so relate the changes in the cell inclusions that any conclusive resemblance to a complete protozoan life cycle is produced.

IMMUNITY

All who have worked with the disease have noted the development of a complete immunity. Löwenthal (1906) says this immunity is shortlived, lasting only one to two months, while others, as Reischauer (1906), say it lasts during the lifetime of the fowl. Still others, as Schmid (1909), say it varies with the severity of the original infection. We are not prepared at present to agree or disagree with any of these observations, as the greatest time between original inoculation and an attempted reinoculation which has elapsed in our series is thirty-five days. Complete immunity was present at the end of that time.

Early Immunity Incomplete.—Reischauer (1906) observes that within the first week following the original inoculation there is no immunity, but that later than this a reinfection runs a very light course or is negative. Marx and Sticker (1902) found the immunity complete after twelve days.

Our results, which confirm these latter observations, are given in the following table:

TABLE II

Time between inoculations	Nature of lesions produced by second inoculation	Result of second inoculation	Number of trials
3 days	Lesions unaltered. Course of disease exactly like that produced by 1st inoculation	Positive	12
6 days	Lesions produced and course of disease changed, but little if at all from normal	Positive	9
9 days	Lesions produced small course of disease shortened and very mild.	Positive	8
12 to 14 days		Negative	15
35 days		Negative	6

Immunity Specific.—The above results were all obtained from a considerable number of cases and show a remarkable agreement. In the cases that were found to be immune after the twelfth day, repeated inoculations, with different strains of virus, were made, but no positive results were obtained. However, in a number of these hyper-immune fowls typical roup was produced by inoculation with another strain of roup-infected virus. Fally (1908) has observed that an immunity to epithelioma contagiosum does not include an immunity to roup, and has argued, therefrom, that each disease is an independent clinical entity. Our results bring us to the same conclusion, and this seems to us one of the strongest reasons for believing the two diseases are due to two distinct causative agents. Seventy-three chickens in all were inoculated. All but ten of these were inoculated with virus which was kept in a test tube, plugged with cotton, from May, 1906, to July, 1910, or with the same strain of virus after one, two, three, or more passages through roup-free fowls. As mentioned above, in some cases the tongues and hard palates were scarified and virulent material rubbed in. In these cases and in about half those in which the comb and wattles alone were inoculated, yellow caseous patches appeared on the mucous membranes of the mouth and throat. However, these patches were well circumscribed in all cases and neither in extent, nor appearance were they typical of roup. Upon examination under the microscope, in the early stages before secondary infection had produced necrotic changes, it was possible to make out changes corresponding exactly to those produced in the epithelium of the comb. There was an hyperplasia of the epithelial layer of the mucous membrane and cell inclusions of the same nature as those seen in the epithelial growths. At no time, so far as we are aware, has any one seen these inclusions in the lesions produced in the mouth by roup. After a few days these patches become necrotic and were made up of epithelial debris and one was unable to longer make out any definite structure. The ten chickens, above referred to, were inoculated with virus freshly received from The College of Hawaii, Honolulu, and the lesions of epithelioma contagiosum differed in no way from those produced by our other virus.

Development of Roup in Immune Fowls.—On the same day in which the ten normal fowls were inoculated with the Hawaiian virus, ten hyper-immune fowls were inoculated with the same virus. These immune fowls developed no lesion of epithelioma contagiosum at any time. However, the normal ones developed typical growths of epithelioma contagiosum at the seat of inoculation, in the usual time, and a few days later three of them developed typical symptoms of roup. There was profuse purulent discharge from the nose and mouth, the sinuses under the eyes became greatly enlarged, the eyes were swollen, closed and filled with cheesy, white exudate, the fowls refused food, became moribund and died. Within a short time another developed the same symptoms and at the same time two of the immune group, which had given no evidence of epithelioma contagiosum after repeated inoculations, succumbed with typical symptoms of roup. The remainder of those inoculated with the Hawaiian virus recovered in the usual way, without showing any signs of roup. However, since Ward (1905) has shown how difficult it is to transmit roup by inoculation (only one of seventeen being positive in his series), its absence from some of the members of the later group would seem to have no bearing on our conclusions. Our results point rather strongly to the conclusion that we had one strain of the virus of epithelioma contagiosum which was free from roup, and one which was mixed with the virus of roup. Because of our results, we are led to believe that Schmid and others who have apparently obtained both diseases with a single virue, had a mixture of the causative agents of two entirely separate diseases.

POSSIBILITY OF DOUBLE INOCULATION

That the virus of epithelioma contagiosum in the form of the scabs is capable of carrying a second infective agent, is shown by a small series of inoculations we carried out in 1910. Two of the cockerels used had slight purulent discharges from the nostrils, having what is usually termed a cold. These cockerels were inoculated with epithelioma contagiosum and later material was taken from them and four other cockerels inoculated. Each

of these four developed a cold exactly similar to the one present in the original hosts, in addition to the lesions of epithelioma contagiosum. Eight other cockerels inoculated with the same virus except that it had been passed through healthy cockerels, were entirely free from any symptoms of a cold.

COMPLEMENT FIXATION

From the fact that an immunity is developed, it occurred to us that during the course of the disease there might be a specific antibody developed in the blood. Following this idea we carried on complement fixation tests on the blood of normal and infected fowls.

Preparation of Material.—After the usual manner, rabbits were immunized against the red blood cells of sheep, and the haemolytic amboceptor thus obtained titrated and preserved in a dark place. An antigen was prepared from the tumors produced on the combs of fowls by epithelioma contagiosum, and from the liver of a fowl having epithelioma contagiosum, after the method of Michaelis and Lisser. Fresh complement or alexin was obtained daily by bleeding guinea pigs. Blood was taken from a sheep every other day, defibrinated, centrifuged, and the corpuscles washed four times in physiological salt solution. Blood was taken from normal and diseased fowls under as nearly as possible aseptic conditions and allowed to stand on ice for from 12 to 24 hours. The clear serum was then pipetted off, and inactivated for 30 minutes at 57° C. in a water bath. After careful titration of the alexin amboceptor and antigen, the factors of our haemolytic system were now assembled and were mixed according to table No. 3.

Technique of Bleeding Fowls.—The technique used in obtaining the blood from the fowls may be of interest, because we have not been able to find a similar method described in the literature, and it was only after trying several unsatisfactory methods that we hit upon this method. It is not necessary to kill the fowls, as considerable quantities of blood can be taken without damaging the bird, and they can be bled rather frequently as they show marvelous powers of recuperation. Even

when a fowl is bled to the point of weakness, he soon recovers and after a lapse of a few days seems to have nearly replaced the lost blood. The wing was raised from the body, and the feathers plucked from the under surface which was then cleaned with 1% tricresol. The fowl was laid upon its side, being held down by the left forearm of the operator while the upper wing was grasped and extended backward by the left hand. Then with small, sharp-pointed scissors, a small incision was made in the brachial artery or vein just where it passes over the flexor surface of the elbow. If only a small quantity of blood was wanted, the vein was opened; while if a larger quantity was needed, the artery was opened. As the vessel was opened the blood welled up into the natural receptacle formed by the elbow and the membrane of the wing. As this anatomical cup filled the blood was drawn up into a sterile Luer hypodermic syringe, without any needle attached, and immediately transferred to a small sterile test tube. A glass Luer syringe is preferable, because it can readily be operated by one hand, and is also readily cleaned, when it is desired to bleed a number of fowls. By following this method, it was found that 5 c.c. or even more, of blood could be taken from a full-grown fowl without causing it any apparent inconvenience. Even when the artery is opened the haemorrhage stops very soon, because of the great coagulability of the blood, and if it does not do so the operator can readily stop it by fairly strong flexion of the wing at the elbow. In a long series of bleedings only two fowls were lost, one from excessive bleeding, and one from an infection of the wing leading to gangrene.

TABLE III

TABLE OF COMPLEMENT FIXATION DATA					AMOUNTS IN CUBIC CENTIMETERS	
I					II	
	Na Cl	Suspected serum	Antigen	Alexin ¹	Amboceptor	Sheep cells, 5% suspension ²
1	1	0.1	1	1 cc.	1 cc.	1 cc.
2	2	0.1		1 cc.	1 cc.	1 cc.
3	1	0.2	1	1 cc.	1 cc.	1 cc.
4	2	0.2		1 cc.	1 cc.	1 cc.
5	1		1	1 cc.	1 cc.	1 cc.
6	..		2	1 cc.	1 cc.	1 cc.
7	2	..		1 cc.	1 cc.	1 cc.
8	2		1		1 cc.	1 cc.
9	3				1 cc.	1 cc.
10	4					1 cc.

¹ Incubate one hour after thorough shaking.

² Incubate 5 to 10 hours after thorough shaking.

Variations in Amounts of Suspected Serum Used.—By adding additional tubes corresponding to tubes 1 and 2, 3 and 4, the quantities of the suspected serum were varied, being added in all quantities from 0.1 to 0.8 c.c. The antigen was found no longer to prevent haemolysis at a dilution of 1 to 100, so was used at a dilution of 1 to 200. The alexin usually produced complete haemolysis at a dilution of 1 to 20 or 1 to 25. The amboceptor was used at a dilution of 1 to 1000, and the sheep cells in a 5% suspension in physiological salt solution.

Complement Fixation.—With normal serum in quantities from 0.1 ccm. up to 0.8 ccm. haemolysis was complete. Haemolysis was usually complete after two hours, and always at the end of five hours' incubation. The same result was obtained with serum taken from infected fowls during the period of incubation. However, a different result was obtained with serum from fowls in which the lesions of epithelioma contagiosum were present. In this serum there was considerable evidence of a specific antibody. Haemolysis did not occur as readily and was almost constantly incomplete at the end of ten hours incubation. The results were not as striking as we believe they could be made by variation in the nature of the antigen, and since there is no question of diagnosis involved will never be of practical value, but it is cer-

tainly of interest as denoting the formation of a specific antibody, or a deviation of complement, in the serum of the infected fowl by an infection of epithelioma contagiosum.

It is also of interest to note, in this connection, that antibodies are most strongly developed in the presence of protozoan infections and that this evidence, in so far as it goes, tends to support the protozoan hypothesis as to the etiological factor in epithelioma contagiosum.

Appearance of Specific Antibody.—While blood was taken from four or five diseased fowls almost daily for a period of almost four weeks, we are not prepared to say definitely at just what stage of the disease the antibody is first present. However, we were unable to find it present to any marked degree until the lesions were fairly well marked, except in those where the method of inoculation was intravenous injection, when it seemed to appear earlier. In any haemolytic system, the addition of normal serum may at times produce fixation, but that this was not so in our series is shown by the contrast between the amount of fixation in tubes, one of which contained serum from a normal healthy fowl, and another of which contained an equal amount of serum from an infected fowl taken under exactly the same conditions. Each set was carefully controlled (see table 3) and on every occasion when the controls indicated that none of the factors of the haemolytic system was at fault, there was uniformly markedly more fixation in the tubes containing serum from fowls having epithelioma contagiosum than in those having like amounts of normal fowl serum. The following are the results of a single day's experiment and are given because they are typical of the results obtained on many days. The numbering of the tubes corresponds to that in table 3.

TABLE IV

1. Serum from normal fowl.

No.	Amount of serum in ccm.	Result
1	0.2	Haemolysis complete
2	0.2	Haemolysis complete
3	0.4	Haemolysis complete
4	0.4	Haemolysis complete
5	0.6	Haemolysis complete
6	0.6	Haemolysis complete

2. Serum from fowl fifteen days after inoculation (lesions on comb well developed).

No.	Amount of serum in ccm.	Result
1	0.2	Some sediment, supernatant fluid cloudy, marked contrast to normal
2	0.2	Haemolysis complete
3	0.4	Considerable fixation
4	0.4	Haemolysis complete
5	0.6	Considerable fixation, marked when contrasted with No. 5, table 1
6	0.6	Slight fixation

3. Serum from fowl eight days after inoculation on comb, lesions extensive, about half as thick as a pea.

No.	Amount of serum in ccm.	Result
1	0.2	Considerable fixation
2	0.2	Nearly clear, haemolysis complete
3	0.4	Considerable fixation
4	0.4	Haemolysis complete

4. Serum from fowl ten days after inoculation on the comb.

No.	Amount of serum in ccm.	Result
1	0.2	Complete haemolysis
2	0.2	Complete haemolysis
3	0.6	Marked fixation
4	0.6	Nearly complete haemolysis

5. Serum from fowl six days after intravenous injection of 1 ccm. of a physiological salt emulsions of ground scabs

No.	Amount of serum in ccm.	Result
1	0.2	Complete haemolysis
2	0.2	Complete haemolysis
3	0.4	Considerable fixation
4	0.4	Complete haemolysis
Controls Nos. 5, 6 and 7—Complete haemolysis		
Controls Nos. 8, 9 and 10—No haemolysis		

It would be interesting to compare the haemolytic reactions of the serum from fowls having epithelioma contagiosum and those having roup. Our intention was to do this, but time did not permit. That the fowls used for all our experiments were entirely free from the effects of any former infection of either epithelioma contagiosum or roup was assured by using fowls raised at the University Farm where neither disease exists.

CONCLUSIONS

1. Epithelioma contagiosum is a specific infectious disease.

(a) The virus is constantly present in material from the lesions found on the head and on the buccal mucous membranes, and in the blood of infected fowls. The disease is readily and constantly produced by inoculation with material from the lesion or with the blood from infected fowls. This inoculation is not the transplantation of tumor cells from one fowl to another, as the virus is present in the filtrate after passage through a Berkfeld filter, and so far as we are able to discover neoplasms have not been produced by inoculation with such filtered extract, with the exception in one case of a sarcoma of the fowl transmissible by an agent separable from the tumor cells as described by Rous (1911).

(b) The period of incubation varies from three to twelve days depending on the virulence of the virus and on the method of inoculation.

(c) The virulence of the virus is lowered by age and by the action of chemicals. Within limits the virulence of the virus increases with passage through a fowl.

(d) An immunity is produced, which is complete within a definite time, is specific and of considerable duration.

(e) The tissue reaction at the point of inoculation is very similar to that produced by inoculation with known infectious agents.

(f) There is a definite relation between the resistance of the host and the virulence of the infection, *i.e.*, an inoculation with a virus of reduced virulence produces a reaction that is entirely local, while a more virulent strain produces a reaction that is not limited to the point of ingress.

(g) In response to an inoculation with epithelioma contagiosum there is produced a specific antibody in the blood of the host.

2. Epithelioma contagiosum and roup are entirely independent diseases.

(a) Epithelioma contagiosum is constantly and readily transmitted by inoculation, while roup is not (Ward).

(b) Immunity conferred by an inoculation with the virus of epithelioma contagiosum does not prevent the contraction of roup.

(c) Under the conditions of our experiment, epithelioma contagiosum was not fatal to mature fowls, while all that contracted roup died.

3. The cell inclusions present in the hyperplastic epithelial cells of epithelioma contagiosum show changes which may perhaps represent stages in the development of a protozoan parasite, but we are unable to so connect them that a complete life-cycle is definitely established.

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